

Progress report validation of parameters to determine unconsciousness during slaughter of veal calves

M.A. Gerritzen, M.T.W. Verhoeven and V.A. Hindle

LIVESTOCK RESEARCH
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Authors
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Wageningen UR Livestock Research

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Preface

Arising from the debate concerning the welfare of animals before and at slaughter, the Dutch Ministry of Economic Affairs ratified a motion on ‘Improving the welfare of livestock designated for slaughter’. Furthermore, in the covenant on slaughter without stunning, the Dutch Ministry of Economic Affairs has, in close compliance with concerned parties, listed areas requiring further research to help safeguard and improve animal welfare during unstunned slaughter (Agreement on unstunned slaughtering in accordance with religious rites, June 2012). These areas include:

- Identification of critical control points
- **Validation of parameters to examine unconsciousness,**
- Reduction in time between neck cut and loss of consciousness,
- Number of neck cuts required,
- Method and duration of restraint.

One of the areas of concern involves the assessment of unconsciousness, as this is mandatory when following the EU Council Regulation 1099/2009, in both stunned and unstunned slaughter of animals. In this progress report we present the results of a study into the validation of parameters to assess unconsciousness in stunned (captive bolt) and unstunned veal calves.

Summary

Introduction: The Dutch Ministry of Economic Affairs ratified a motion on "Improving the welfare of livestock designated for slaughter". One of the areas of concern involves the assessment of unconsciousness in both stunned and unstunned animals, as is mandatory following the EU Council Regulation 1099/2009. However, the validity of certain behavioral parameters to assess unconsciousness under different stunning and slaughter conditions is under (inter)national debate.

Research question: The aim of this study was to validate the absence of the righting reflex, rhythmic breathing, threat-, withdrawal-, cornea-, and eyelid reflex as parameters to assess unconsciousness in veal calves subjected to different stunning and slaughter methods.

Material and methods: At a commercial slaughter plant in The Netherlands calves (201 ± 22 kg) were randomly assigned to one of the following four treatments: 1. Captive bolt stunning followed by neck cutting (n=25); 2. Unstunned slaughter in an upright position (n=7); 3. Unstunned slaughter in an inverted position (180° rotation) (n=25); 4. Unstunned slaughter in an upright position followed by captive bolt stunning at 40 s post neck cut (n=25). Each calf was equipped with non-invasive EEG electrodes prior to the procedure. All reflexes were verified once in a controlled conscious state.
After start of the procedure (T=0 s) reflexes were assessed on T=5 s for treatments 1 and 2 and from T=15 s every 20 s for all treatments until all reflexes showed a negative response three times in a row and a flat EEG was observed. Visual assessment of EEG traces was used to determine loss of consciousness. Timing of loss of consciousness was related to timing of loss of reflexes.

Results and conclusions
In both stunned and unstunned calves, unconsciousness was induced and absence and presence of reflexes was linked to EEG activity. Observation of reflexes to assess unconsciousness can be readily applied in daily practice, but EEG recordings are required to validate such reflexes.

Based on EEG activity, all calves lost consciousness immediately or quickly after captive bolt stunning. Loss of consciousness was confirmed by absence of threat-, withdrawal,- cornea,- and eyelid reflexes at 5 s post stunning.

Post-cut captive bolt stunning resulted in immediate loss of consciousness and was as effective as pre-cut captive bolt stunning.

All calves slaughtered without stunning in an upright position were considered unconscious at, on average, 109 s post-cut. Calves slaughtered without stunning in an inverted position were considered unconscious at, on average, 49 s post-cut.
In this study, unstunned slaughter in an upright position resulted in a delay of on average 50 s to loss of consciousness compared with unstunned slaughter in an inverted position.

The righting reflex is not an applicable parameter to assess unconsciousness while animals are fully restrained in a stunning box. Since restraining is mandatory until the animals are unconscious, the righting reflex should not be used to assess state of (un)consciousness during unstunned slaughter, prior to allowing the animal to be hoisted onto the slaughter line.

Determining rhythmic breathing in animals with a cut trachea, restrained in a stunning box, is not possible. Therefore, under the above mentioned conditions, rhythmic breathing is considered not to be an applicable parameter for assessment of (un)consciousness in unstunned slaughtered animals.

In general, but specifically at 40 s after neck cut, absence of the threat- or withdrawal reflex did not indicate unconsciousness. Moreover, the majority of the animals did not respond to a pain stimulus following the neck cut when they were adjudged to be conscious based on EEG activity. The response

to a threat is often difficult to determine due to the amount of blood flowing over the eyes of inverted animals and was also observed to be absent in animals adjudged to be conscious based on EEG activity.

The cornea-, or eyelid reflex was often present when calves were considered unconscious, based on EEG-activity. Presence of the eyelid-, or cornea reflex at 40 s therefore did not indicate consciousness. Absence of the eyelid-, or cornea reflex on the other hand reflected unconsciousness, but both reflexes appeared distinctly conservative parameters of unconsciousness since they disappeared while animals lost consciousness for periods of up to two minutes.

Considering the parameters chosen to assess unconsciousness at slaughter it can be concluded that;

- the righting reflex and rhythmic breathing are difficult to determine under practical conditions,
- the threat-, and withdrawal reflex (reaction to pain stimulus) are invalid parameters in the assessment of (un)consciousness in unstunned veal calves,
- the eyelid-, and cornea reflex provide a very conservative confirmation, but when absent they are a true indicator of unconsciousness.

Samenvatting

Inleiding: Het Nederlandse Ministerie van Economische Zaken heeft een motie over de "Verbetering van het welzijn van slachtdieren" aangenomen. Eén van de aandachtsgebieden in deze motie omvat de beoordeling van bewusteloosheid in zowel bedwelmde, alsmede onbedwelmd dieren, zoals aangegeven is in de verordening 1099/2009 over de bescherming van dieren bij het doden. Echter, er is (inter)nationaal discussie over de geldigheid van bepaalde gedragsparameters waarmee onder verschillende omstandigheden bewusteloosheid wordt beoordeeld.

Onderzoeksvraag: Het doel van deze studie was om de afwezigheid van de oprichtreflex, ritmische ademhaling, dreig-, pijn-, cornea- en ooglid reflexen te valideren als parameters voor het beoordelen van bewusteloosheid in vleeskalveren onder verschillende bedwelmings- en slachtmethoden.

Materiaal en methoden: Op een Nederlandse slachterij werden kalveren (201 ± 22 kg) willekeurig toegewezen aan één van de volgende vier behandelingen: 1. Verdoven door middel van een schietmasker gevolgd door aansnijden (n = 25); 2. Niet-verdoofd slachten in een rechtopstaande positie (n = 7); 3. Niet-verdoofd slachten in rugligging (180° rotatie van het kalf) (n = 25); 4. Niet-verdoofd slachten in een rechtopstaande positie, gevolgd door verdoven door middel van een schietmasker op 40 s (n = 25). Elk kalf werd, voorafgaand aan de procedure, uitgerust met EEG-elektroden. Alle reflexen werden eenmalig gecontroleerd voor start van de procedure. Na het begin van de procedure (T = 0 s) werden alle reflexen beoordeeld op T = 5 s voor de behandelingen 1 en 2 en vanaf T = 15 s om de 20 s voor alle behandelingen tot alle reflexen driemaal achter elkaar negatief waren en daarnaast een vlak EEG werd waargenomen. Visuele beoordeling van het EEG signaal werd gebruikt om het moment van bewustzijnsverlies te bepalen. De tijd tot bewustzijnsverlies werd daarna gerelateerd aan de tijd tot het verlies van de verschillende reflexen.

Resultaten en conclusies
Zowel bij bedwelmde als niet-bedwelmde kalveren werd bewusteloosheid geïnduceerd door de verschillende behandelingen en de af- en aanwezigheid van reflexen werd gerelateerd aan EEG activiteit. Het gebruik van reflexen om bewusteloosheid te beoordelen is gemakkelijk toe te passen in de praktijk, maar EEG activiteit moet worden gebruikt om dergelijke reflexen te valideren.
Alle kalveren verloren onmiddellijk of zeer snel na verdoving door het schietmasker het bewustzijn, gebaseerd op EEG-activiteit. Verlies van bewustzijn werd daarnaast bevestigd door de afwezigheid van de dreig-, pijn-, cornea-, en ooglid reflex op 5 s na verdoving.

Verdoving door het schietmasker op 40 s na onverdoofd aansnijden in een rechtopstaande positie (behandeling 4) leidde eveneens tot het onmiddellijk intreden van bewusteloosheid. Gebruik van het schietmasker in deze groep dieren was even effectief als wanneer dieren verdoofd waren door middel van een schietmasker, gevolgd door aansnijden (behandeling 1).

Niet-verdoofd slachten in een rechtopstaande positie leidde tot het intreden van bewusteloosheid gemiddeld 109 s na aansnijden. Niet-verdoofd slachten in rugligging leidde tot het intreden van bewusteloosheid gemiddeld 49 s na aansnijden. In deze studie leidde niet-verdoofd slachten in een rechtopstaande positie tot een significante vertraging (gemiddeld 50 s) in het verlies van bewustzijn in vergelijking met niet-verdoofd slachten in rugligging.

De opricht reflex is geen parameter die gebruikt kan worden om bewusteloosheid te beoordelen, omdat de dieren volledig gefixeerd zijn. Ritmische ademhaling is geen parameter die gebruikt kan worden om bewusteloosheid te beoordelen, omdat de dieren volledig gefixeerd zijn en de luchtpijp is doorgesneden. Aangezien fixatie verplicht is totdat de dieren bewusteloos zijn, moeten de opricht reflex en ritmische ademhaling niet worden gebruikt als parameters voor het beoordelen van bewustzijn of bewusteloosheid bij het slachten van vleeskalveren.

Een meerderheid van de kalveren reageerde niet op de pijnprikkel na te zijn aangesneden. Deze dieren waren, gebaseerd op EEG activiteit, nog wel bij bewustzijn. De reactie op de dreig reflex is vaak niet zichtbaar door de hoeveelheid bloed die in de ogen stroomt wanneer de dieren in een rugligging zijn gepositioneerd. Een meerderheid van de kalveren reageerde tevens niet op de dreig reflex na te zijn aangesneden. Deze dieren waren, gebaseerd op EEG activiteit, nog wel bij bewustzijn. De afwezigheid van een dreig- of pijn reflex geven niet aan of een dier bewusteloos is. Dit geldt specifiek op 40 s na de halssnede.

De cornea- of ooglid reflex was vaak aanwezig in kalveren die bewusteloos waren, gebaseerd op EEG-activiteit. Aanwezigheid van de eyelid-, of cornea reflex op 40 s hoeft dus geen bewustzijn aan te duiden. Wanneer de cornea- of ooglid reflex afwezig waren, dan waren kalveren bewusteloos, gebaseerd op EEG-activiteit. Zowel de cornea- als ooglid reflex zijn echter (te) conservatieve parameters voor bewusteloosheid, omdat ze altijd verdwenen, tot wel 2 min, nadat kalveren het bewustzijn hadden verloren.

Met betrekking tot de parameters voor het beoordelen van bewusteloosheid in vleeskalveren kan het volgende worden geconcludeerd:

- De opricht reflex en ritmische ademhaling zijn niet te beoordelen onder de huidige praktijkomstandigheden,
- Afwezigheid van de dreig- of pijn reflex (reactie op pijnprikkel) zijn geen geldige parameters voor het beoordelen van bewusteloosheid in niet verdoofde vleeskalveren,
- Afwezigheid van de cornea- en ooglid reflex zijn conservatieve parameters in de beoordeling van bewusteloosheid in vleeskalveren. Bij afwezigheid van deze twee reflexen kan bewusteloosheid worden aangenomen.

1 Introduction

Arising from the debate concerning the welfare of animals before and at slaughter, the Dutch Ministry of Economic Affairs ratified a motion on “Improving the welfare of livestock designated for slaughter”. Furthermore, in the covenant on unstunned slaughter, the Dutch Ministry of Economic Affairs has, in close compliance with concerned parties, listed areas requiring further research to help safeguard and improve animal welfare during slaughter without stunning (Agreement on unstunned slaughtering in accordance with religious rites, June 2012). These areas include:

1. Identification of critical control points,
2. **Validation of parameters to examine unconsciousness,**
3. Reduction in time between the neck cut and loss of consciousness,
4. Number of neck cuts required,
5. Method and duration of restraint.

One of the areas of concern involves the assessment of unconsciousness in both stunned and unstunned animals, as is mandatory following EU Council Regulation 1099/2009.
Animals are stunned prior to slaughter in order to minimize pain and distress. Stunning however, is not always compatible with particular methods of slaughter prescribed by religious rites and is not mandatory in animals subjected to this type of slaughter. Post-cut stunning is practiced in some European countries to improve animal welfare compared to no stunning at all (Farouk 2013). Assessment of unconsciousness and insensibility is a legal requirement during the slaughter process (EU Council Regulation 1099/2009, 2009). During unstunned slaughter, animals are restrained and bled through a transverse incision of the neck, severing the skin, muscles, trachea, oesophagus, carotid arteries, jugular veins and major nerves. As a consequence there is a severe decrease in cerebral blood flow leading to a rapid onset of disorganized brain function and thus unconsciousness (Mellor, Gibson and Johnson, 2009). In cattle, consciousness after the neck cut is prolonged, because the vertebral arteries, not severed by the neck cut, supply blood to the circle of Willis and maintain a blood supply to the brain (Baldwin and Bell, 1963). It remains unclear how long it takes for cattle to lose consciousness during slaughter without stunning, since studies using EEG provided contradictory results. Some studies suggest an almost instant loss of consciousness with little variation among individuals while other studies report loss of spontaneous brain activity 75 ± 48 s post neck cut (range 19-113 sec) with possibly intermittent sensibility for up to 123 to 323 s after slaughter (Daly, Kallweit, and Ellendorf, 1988; Newhook and Blackmore, 1982). There is substantial (inter)national debate on which parameters most adequately assess unconsciousness at slaughter and merit further investigation (EFSA 2014). The use of recorded brain activity (as presented in an electroencephalogram or EEG) is considered to be the most objective method for assessing unconsciousness and is generally accepted as the ‘golden standard’ (Verhoeven et al. 2015; EFSA 2014, Erasmus, Turner and Widowski, 2010). Due to the complexity of collecting EEG data at slaughter, absence of reflexes and other behavioral parameters are often used to assess unconsciousness, including rhythmic breathing, cornea, withdrawal, threat and eyelid reflexes. There is, however, a scarcity of scientific publications reporting a correlation between unconsciousness ascertained by EEG activity and behavioral parameters of unconsciousness that could be used in slaughterhouse conditions in bovines. It has been decided in The Netherlands, that animals subjected to slaughter without stunning should be unconscious within 40 s, based on absence of at least 3 of the following 5 parameters: 1. rhythmic breathing; 2. (spontaneous) eyelid reflex 3. withdrawal reflex; 4. righting reflex; 5. threat reflex. Different studies have shown that the absence of the cornea- and eyelid reflex and rhythmic breathing were distinctly conservative parameters when assessing unconsciousness in unstunned slaughter of sheep (Gerritzen et al. 2014) and veal calves (Lambooij et al. 2012). On the contrary, absence of the withdrawal- and threat reflexes did not reflect unconsciousness after unstunned slaughter in sheep (Gerritzen et al. 2014). The objective of the current study was to determine absence or presence of the following parameters: righting reflex, rhythmic breathing, eyelid-, cornea,-withdrawal-, and threat reflexes in relation to (un)consciousness as identified by EEG activity during stunned and unstunned slaughter in veal calves.

2 Material and Methods

2.1 Experimental set-up

Animals subjected to slaughter (with or without stunning) were observed during 7 days at an abattoir in The Netherlands from September to November 2014. Eighty-two cattle of mixed breeds (warm carcass weight 201 ±22 kg) were randomly selected from groups in lairage and held individually in a rotation box (Nawi, Borculo, The Netherlands, picture 1) while equipped with five EEG electrodes. Animals were restrained in the V-shaped restraining box without a belly supporting plate. The V-shape of the restraining semi-supported the standing or upright position.

Experiments and slaughter conditions were approved by the ethical committee of the institute and by the competent authorities.



Picture 1: restraining box

Animals were randomly subjected to the following four treatments groups;

Captive bold stunning (Cash Magnum 9000s) followed by neck cut within 3s, (n=25)
Unstunned slaughter in an upright (standing) position, (n=7)
Unstunned slaughter in an inverted (180 ° rotation) position, (n=25)
Unstunned slaughter in an upright (standing) position, followed by captive bolt stunning at 40 s, post neck cut (n=25)

Calves subjected to the above treatments were observed during 7 days at a commercial abattoir in The Netherlands from September to November 2014. In total 82 cattle of mixed breeds (warm carcass weight 201 ± 22 kg) were randomly selected from groups in lairage and held individually in a rotation box (Nawi, Borculo, The Netherlands) while equipped with EEG electrodes. The rotation box was operated by plant personnel who normally operate the restraining device. Calves were randomly assigned to one of the four treatments listed above. Treatment group 3 was limited to 7 calves because of animal welfare concerns: i.e. delayed induction of unconsciousness due to insufficient bleeding. All calves were restrained with a head yoke and chin lift, but without a belly support plate (not available on this rotary box model). The neck cut was performed as a clean incision through the structures at the front of the neck –severing the trachea, oesophagus, carotid arteries and jugular veins. All stunning and slaughter procedures were performed by a skilled halal slaughter man.

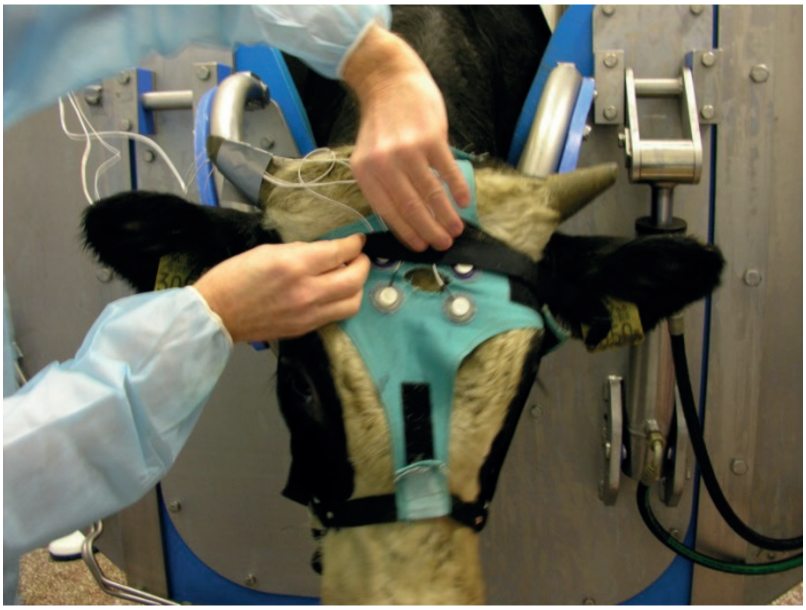
The design of the restraining box made it impossible to observe breathing movements of the chest or abdomen.

2.2 EEG recordings

After being placed in the restrainer, the head of the animal was shaved using an electrical hair trimmer (Aesculap favorita II GT104, Braun Suhl GmbH, Germany) to enable placement of EEG electrodes. Thereafter an elastic halter was placed around the calves head. A cotton shaped cross was attached to the halter that had Velcro parts on each ending. In the middle of the cross a hole was cut, through which the captive bolt could be placed. Five rubber sensor carriers were placed around the 'shooting-hole', (picture 2). Five Ag/Cl pellet electrodes were placed in the rubber sensor carriers on the shaved skin of the calve (TMSi, Oldenzaal, The Netherlands). Each electrode was wrapped in a small sponge soaked in heparinised saline solution, which served as interface to the skin. One electrode was placed over the frontal bone, on the sagittal midline on a line extending across between the base of both ears. Two electrodes were placed 2 cm left and right of the sagittal midline and 3 cm frontal from the first electrode, and the other two electrodes were placed 2 cm left and right of the sagittal midline and 6 cm frontal from the first electrode. An elastic band was wrapped around the calves' head once to secure the upper three electrodes, but leaving the ears and eyes free to move (picture 3).

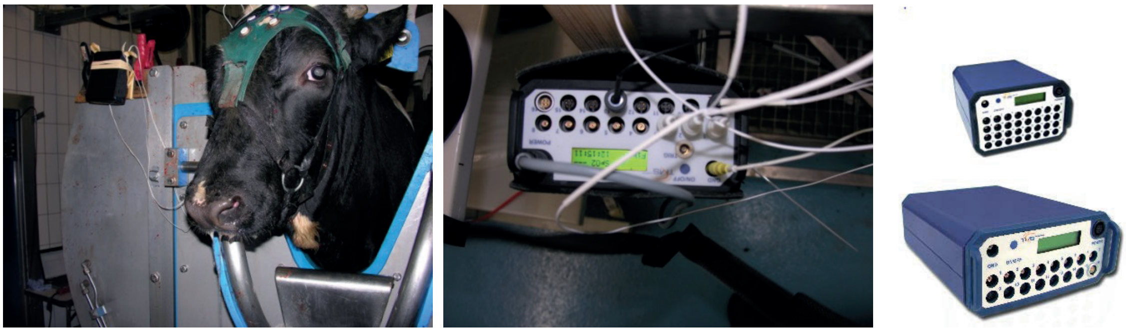


Picture 2: calf equipped with non-invasive EEG electrodes



Picture 3: placement of non-invasive EEG electrodes

All electrodes were connected via a 140 cm active coated cable to the Porti system with 32 channels (Twente Medical Systems International (TMSi), Oldenzaal, The Netherlands, picture 4). Porti uses bipolar amplifier technology with high input impedance (> 1 giga ohm) that amplifies the potential difference between pairs of electrodes. The input amplifier is dimensioned as multichannel instrumentation amplifier. Electrode impedance was <5kΩ. The EEG was displayed with a high and low frequency cut off of 0.5 and 30 Hz, respectively, but uploaded to a computer unfiltered. Sample rate was set at 0.5 kHz. Once the electrodes had been placed properly and a good live signal was obtained, baseline EEG activity was recorded for at least two minutes. At T=0 s, the calf was subjected to one of four treatments as described previously. The EEG was recorded until a flat EEG (< 10% baseline activity) was observed and all reflexes had a negative response three times in a row.



Picture 4: Calf equipped with ECG electrodes connected to recording system (porti system; www.tmsi.com)

2.3 Behavioral parameters and reflexes

The threat-, withdrawal-, eyelid-, and cornea reflexes were verified once in a controlled conscious state prior to stunning or slaughter. After starting the procedure (T=0 s), the reflexes were assessed on T=5 s for treatment 1 and from T=15 s every 20 s for all treatments until all reflexes showed a negative response three times in a row together with observation of a flat EEG. Time to loss of the reflexes was defined as the first moment at which a reflex was determined to be absent three times in a row, since loss and recovery of a reflex was observed in some of the calves. The threat reflex was assessed as the presence of a blinking reaction to an abrupt movement of the index finger towards the eye ball without touching the eye. The withdrawal reflex in response to pain stimuli was assessed by pinching the calves nose between two fingertips and determining whether or not it responded with withdrawal of the nose or head. The eyelid reflex was assessed with a gentle touch of the eyelid, and considered as present when a blinking reaction was observed and absent when no response was observed. The cornea reflex was assessed by a gentle touch of the cornea with the index finger, considered as present when a blinking reaction was observed and absent when no response was observed. Stimuli to trigger responses were performed in a random order for each calf. Righting reflex and rhythmic breathing were verified based on constant observation. The righting reflex is interpreted as an attempt to raise the head or to regain an upright position between stunning and bleeding until release from restraint. Rhythmic breathing is interpreted as maintenance of a normal and active breathing pattern.

2.4 Data analyses

EEG data was displayed, stored and analysed using PolyBench software (TMSi, Oldenzaal, The Netherlands). EEG activity of each calf was visually assessed to determine the beginning of the following stages: EEG-baseline, EEG-transitional, EEG-unconscious and EEG-minimal-brain-activity. The start of each EEG stage was determined visually.

Figure 1a-d shows an example of a representative series of four seconds of EEG registration during these different stages. Baseline EEG consisted of a low amplitude, high frequency signal, indicating alert calves (Fig. 1a). A transitional signal occurred when low frequency, high amplitude (firing of neurons in a synchronized fashion) became more apparent compared to baseline, associated with reduced consciousness (Fig. 1b). When low frequency, high amplitude dominated the EEG trace this indicated unconsciousness (Fig. 1c). Minimal brain activity was reflected by an almost flat signal (<10% of baseline activity) (Fig. 1d).

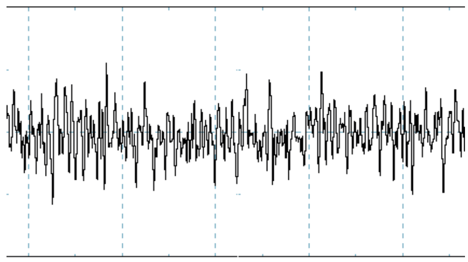


Figure 1a

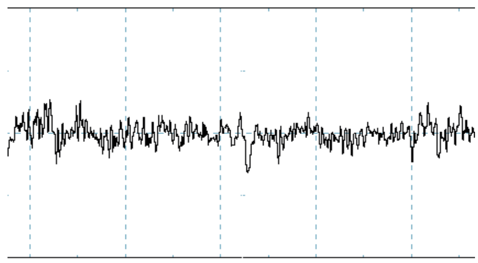


Figure 1b

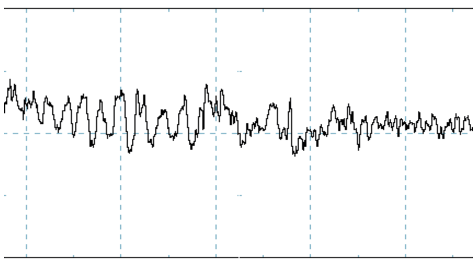


Figure 1c

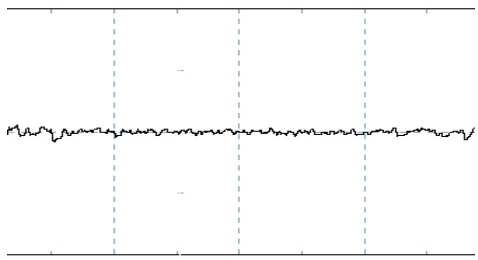


Figure 1d

Figure 1a-d. *Typical examples of the different stages identified with visual assessment of EEG activity prior to and after stunned and unstunned slaughter in calves. The four stages are: baseline (1a; stage 1), transitional (1b; stage 2), unconscious (1c; stage 3), and minimal brain activity (1d; stage 4). Total X-axis represents 5 seconds, Y-axis represents amplitude of the EEG-trace (µV).*

2.5 Statistical analyses

Individual veal calves were considered as the experimental unit in all statistical analyses. Statistical analyses were performed using SAS (version 9.3; SAS Institute, 2004). Normality assays were performed (PROC UNIVARIATE) for all the variables examined. Prior to the analyses, variables lost-eyelid and lost-cornea were log transformed to normalize these variables. Determination of positional (upright or inverted) effect on time to onset of different EEG stages and time to loss of reflexes, were analysed with a GLM procedure according to the model:

$$Y_i = T_i + \varepsilon_i$$

where T_i is Treatment (1 to 4), and ε_{ij} is the residual error term.

Differences were considered to be significant at the 5% probability level.

3 Results

3.1 Animals

The total number of calves used in this study was 82. In one calf from treatment 4, the EEG signal was lost after stunning and we were unable to determine the moment of loss of consciousness. Data from this calf was excluded from further analyses.

3.2 EEG activity

A clear EEG signal was obtained from all calves during baseline recordings. All EEG traces were analysed for typical changes from baseline EEG activity (fig. 1a) towards a transitional EEG (fig. 1b), into an EEG pattern typical of unconsciousness (fig. 1c) ending in a stage of minimal brain activity (fig. 1d).
Calves from treatment 1 (captive bolt stunning) were found to be unconscious (stage 3) from 2 ± 2 s onwards after stunning. No transitional period (stage 2) was observed in these calves. Minimal brain activity (stage 4) was observed from 22 ± 19 s after stunning. One calf lost consciousness (stage 3) 11 s after stunning, according to EEG activity. Excluding this calf, consciousness was lost (stage 3) on average from 1 ± 0 s after stunning. Average duration to onset of minimal brain activity (stage 4) was not affected when excluding this calf from the analysis.

Figures 2a, b and c represent the percentage of calves with different EEG stages through time after slaughter without stunning (T=0 s) in an upright (treatment 2) or an inverted position (treatment 3). At 40 s post neck cut, 20% of the calves from treatment 2 displayed a transitional EEG (stage 2), 0% were considered unconscious (stage 3) and 0% of the calves from treatment 2 had minimal brain activity (stage 4). At 62 s after neck cutting, 80% of the calves from treatment 2 had a transitional EEG (stage 2) which became 100% at 70 s post neck cut. At 132s post neck cut, 80% of the treatment 2 calves were considered unconscious (stage 3) becoming 100% 140 s post neck cut. At 175 s post neck cut, 80% of the treatment 2 group displayed minimal brain activity (stage 4) becoming 100% at 253 s after neck cutting.

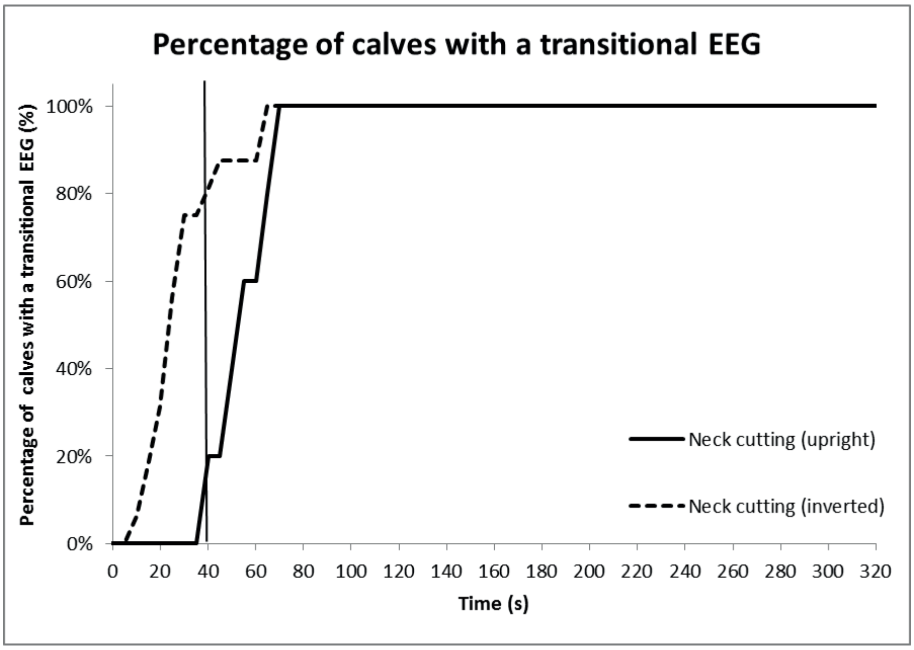


Figure 2a

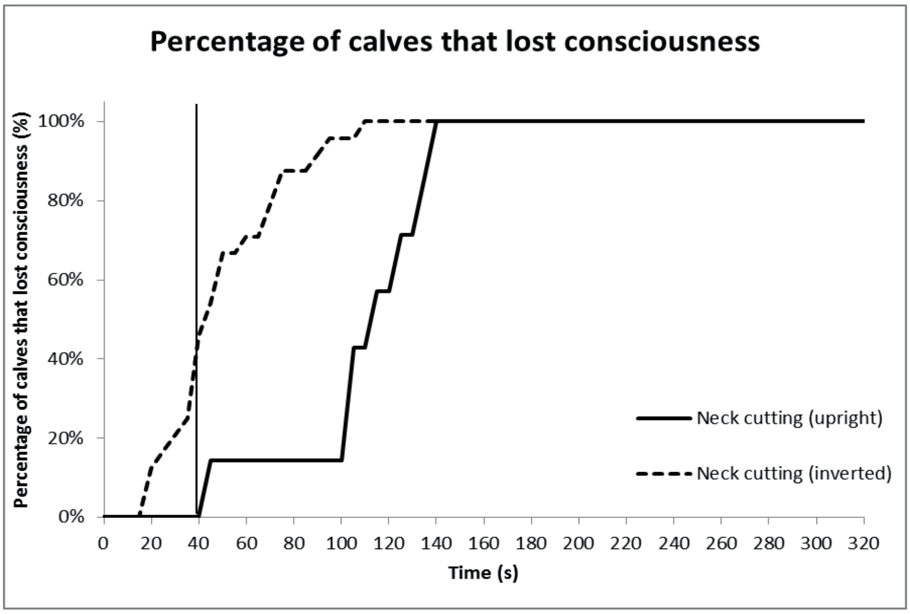


Figure 2b.

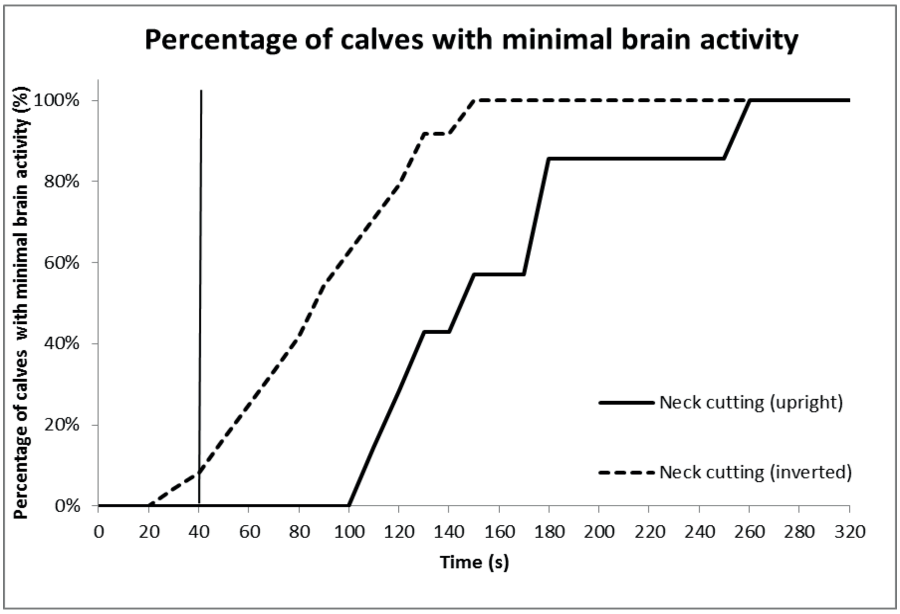


Figure 2c

Figure 2a,b,c. *Percentage of calves with different EEG stages through time after unstunned slaughter (T=0 s.) in an upright (solid lines, n=7) or inverted position (dashed lines, n=25). Vertical black line represents percentage of calves with a specific EEG stage at 40 s.*

At 40 s post neck cut, 81% of the calves from treatment 3 had a transitional EEG (stage 2), 46% were considered unconscious (stage 3) and 8% displayed minimal brain activity (stage 4). Approximately 100% of the treatment 3 group had a transitional EEG (stage 2) at 62 s post neck cut. At 71 s post neck cut, 80% of the calves from treatment 3 were considered unconscious (stage 3) becoming 100% at 109 s post neck cut. At 122 s after neck cutting, 80% of the calves from treatment 3 had minimal brain activity (stage 4) becoming 100% at 147 s post neck cut.

Calves in an upright position (treatment 2), had a transitional EEG (stage 2) on average 27 seconds later (P<0.001) than calves in an inverted position (treatment 3:from 54 ± 13 s and 27 ± 15 s onwards post neck cut, respectively). Upright calves (treatment 2) were considered unconscious (stage 3) on average 50 seconds later (P<0.001), than inverted calves from treatment 3 (from 109 ± 32 and 49 ± 25 s onwards post neck cut, respectively). Minimal brain activity (stage 4) was measured on average 71 seconds later (P<0.001) in upright calves (treatment 2) than inverted calves (treatment 3). Times to stage 4 ranged respectively from 157 ± 50 s and 86 ± 34 s onwards post neck cut.

Calves from treatment 4 (captive bolt stunning in upright position after neck cutting) were stunned on average at 34 ± 8 s post neck cut. At stunning, 67% of the calves had a baseline-like EEG (stage 1), 8% stage 2, 21% stage 3 and 4% stage 4.

3.3 Behavioral parameters and loss of reflexes

In some calves, reflexes disappeared, but were restored later during bleeding. Observation and assessment of the righting reflex was impossible during the period of restraint. Calves were fully restrained (head and body) during the slaughter procedure and righting reflexes of head and or body could not therefore not be expressed and observed during the procedure.

Rhythmic breathing could also not be observed. The design of the restraining box made it impossible to observe breathing movements of the chest or abdomen. Once the trachea had been severed it was not possible to distinguish between pathological and active breathing movements.

Reflexes were tested and present in all calves during baseline recordings. Threat-, withdrawal-, cornea-, and eyelid reflexes were all lost from 5 seconds onwards post captive bolt stunning in calves from treatment 1.

Figures 3a,b,c present the percentage of calves that lost the threat-, withdrawal-, cornea- and eyelid reflexes in relation to the different EEG stages through time after unstunned slaughter in an upright position (treatment 2).

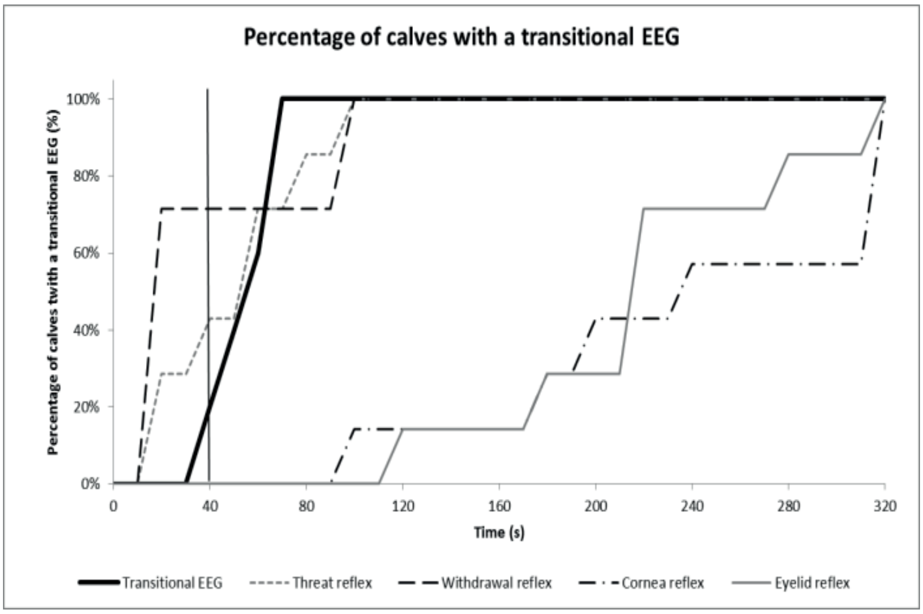


Figure 3a.

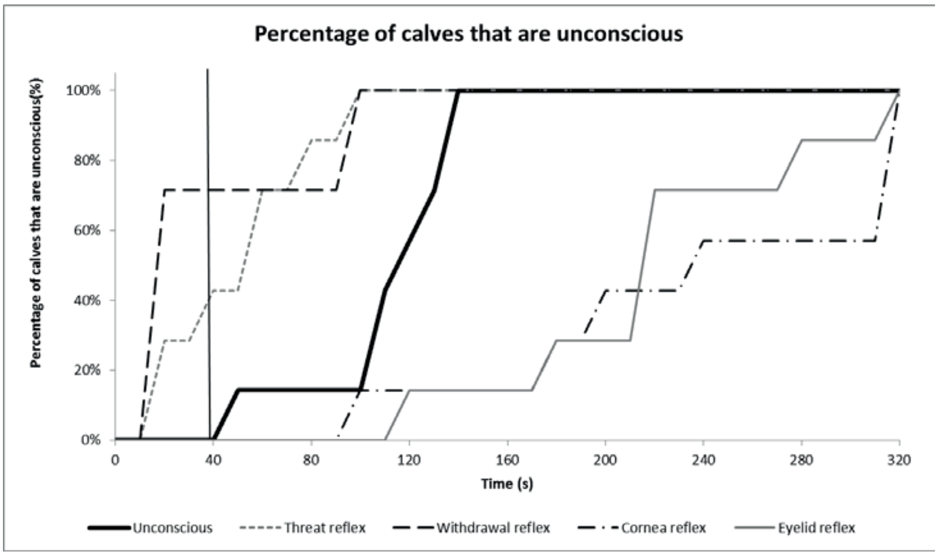


Figure 3b.

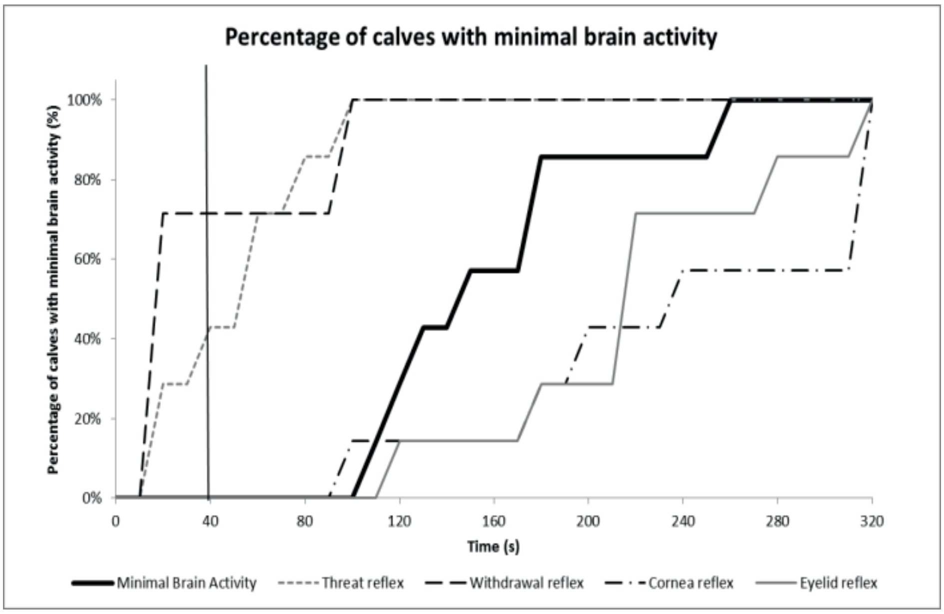


Figure 3c.

Figure 3a,b,c. *Percentage of calves (n=7) that lost the threat-, withdrawal-, cornea- and eyelid reflexes in relation to the different EEG stages through time after unstunned slaughter in an upright position at T=0 s. Vertical black line indicates percentage of calves with a specific EEG stage and loss of reflexes at 40 s.*

The threat-, and withdrawal reflexes were both lost in 100% of the calves before loss of consciousness (stage 3) had been observed. The eyelid reflex was lost in 100% of the calves after minimal brain activity (stage 4) had been observed. The cornea reflex was lost in 14% of the calves after loss of consciousness (stage 3) had been observed and in 86% of the calves after minimal brain activity (stage 4) had been observed.

Figures 4a,b,c present the percentage of calves that lost the threat-, withdrawal-, cornea- and eyelid reflexes in relation to the different EEG stages through time after unstunned slaughter in an inverted position (treatment 3).

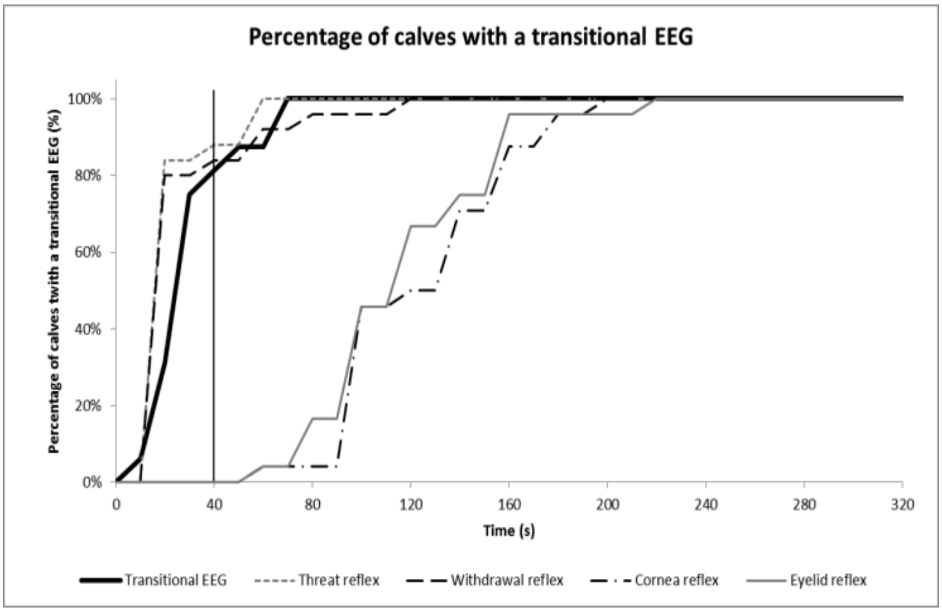


Figure 4a.

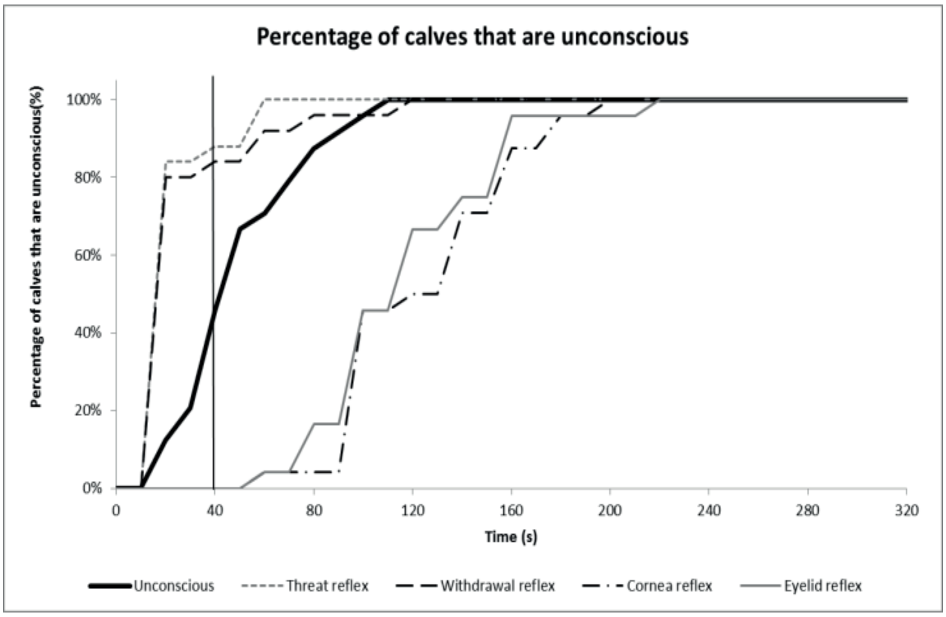


Figure 4b.

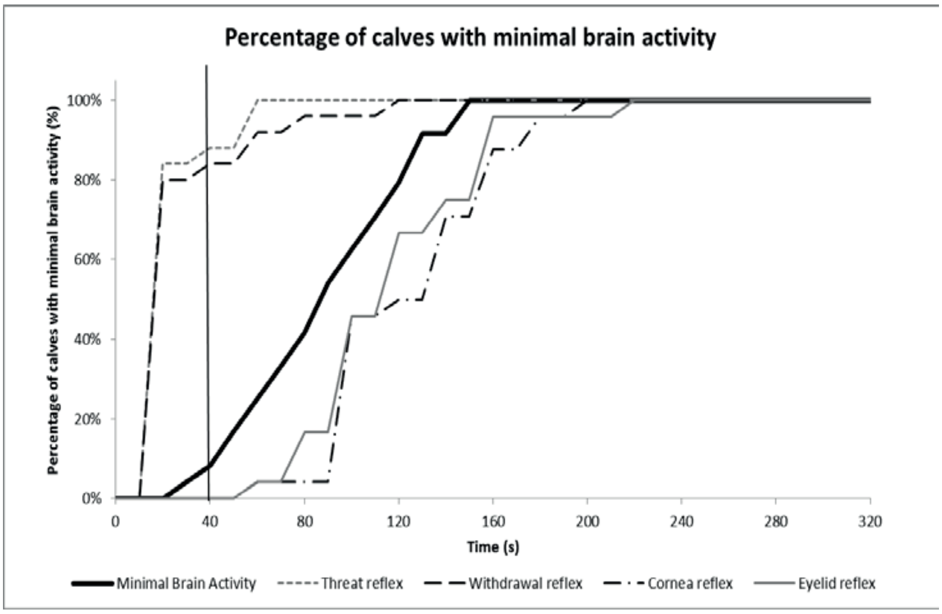


Figure 4c.

Figure 4a,b,c. *Percentage of calves (n=25) that lost the threat-, withdrawal-, cornea- and eyelid reflexes in relation to the different EEG stages through time after unstunned slaughter in an inverted position at T=0 s. Vertical black line indicates percentage of calves with a specific EEG stage and loss of reflexes at 40 s.*

The threat reflex was lost in 100% of the calves prior to loss of consciousness (stage 3). The withdrawal reflex was lost in 96% of the calves before loss of consciousness had been observed (stage 3) and in 100% of the calves before minimal brain activity had been observed(stage 4). Both cornea-, and eyelid reflexes were lost in 100% of the calves after minimal brain activity had been observed at stage 4. Calves from treatment 2 lost the eyelid reflex on average 103 s later ($P<0.001$) than calves from treatment 3 (from 218 ± 65 s and 115 ± 36 s onwards post neck cut, respectively). Calves from treatment 2 lost the cornea reflex on average 112 s later ($P<0.001$) than calves from treatment 3 (from 235 ± 86 s and 123 ± 34 s onwards post neck cut, respectively).

The threat-, and withdrawal reflex were both lost in 100% of the calves from treatment 4 before loss of consciousness had been observed. The eyelid- and cornea reflex were both lost at time of stunning.

4 Discussion

This study was designed and performed to provide insight into the validity of the following behavioral reactions or reflexes that are used to assess unconsciousness at slaughter in calves: righting reflex, rhythmic breathing threat-, withdrawal-, cornea-, and eyelid reflexes. Brain activity, is currently presented with an electroencephalogram or EEG for the assessment of unconsciousness.

Calves were subjected to one of the following procedures: captive bolt stunning followed by neck cut, unstunned slaughter in an upright position or an inverted position and unstunned slaughter in an upright position followed by captive bolt stunning at 40 s. Captive bolt stunning causes an intracranial trauma disrupting electrical activity in the brain. Depending on the method, location and force applied, damage is done to the cerebrum, cerebellum and brainstem and a reduction in blood circulation can be observed (Finnie, 1993; Finnie, 1997). The duration of unconsciousness depends on the extent of the damage to nervous tissue and to which degree the blood supply is reduced (Shaw, 2002; Humane Slaughter Association, 2006). As a result of the trauma, the animal may die, but this is not a guaranteed outcome of stunning and immediate bleeding out of the animal is required (Appelt and Sperry, 2007). After effective captive bolt stunning, an animal should show no reflexes, should not respond to stimuli from the environment and immediate collapse (loss of posture) should be observed (Gregory and Shaw, 2000). Immediate collapse was observed in some of the calves, but firm restraining of the calf often prevented collapse and obstructed observation. At 5, 15 and 35 s post stunning, none of the assessed reflexes were positive in this study, indicating successful stunning of the animals. Rhythmic breathing, spontaneous blinking, blinking reflex, righting reflex, vocalizations, or nystagmus are indicative that the stunning is not irreversible and that there had been a return to a degree of consciousness (Grandin, 2002). Both rhythmic breathing and the righting reflex could not be assessed due to firm restraint of the calf. EEG recordings in animals stunned with a captive bolt showed high-amplitude, slow-frequency waves in theta- and delta-frequency bands post stunning. Indicating immediate unconsciousness, similar to findings in other studies (Zulkifli et al., 2014; Lambooy and Spanjaard, 1981). In the current study, one calf had a period of baseline -like EEG activity after captive-bolt stunning, lasting for 11 s after stun application. All reflexes were lost at 5 s post stunning in this calf. This prolonged period of baseline -like EEG activity may have been caused by incorrect positioning of the stunner, a deflected shot, or incorrect functioning of the stunning device itself.

Unstunned slaughter is based on a neck cut severing both carotid arteries and jugular veins completely, subsequently disrupting the blood supply to the calf's brain. In cattle, compared to other ruminants, consciousness after the neck cut is prolonged, because the vertebral arteries not severed by the neck cut, supply blood to the circle of Willis and play a direct role in the blood supply to the brain (Baldwin and Bell, 1963). There is much variation between unstunned cattle losing consciousness after the neck cut. Both Lambooij et al., 2012 and Daly and Kallweit, 1988 reported loss of spontaneous brain activity on average 80 s post neck cut (range 19-113 s), but Newhook and Blackmore, 1982 suggest possible intermittent sensibility may occur up to 123 to 323 s after slaughter in cattle. In this study, calves lost consciousness, based on EEG activity, at 109 ± 32 s post neck cut when slaughtered in an upright position or 49 ± 25 s when slaughtered in an inverted position. It was also observed that the duration to loss of consciousness in calves slaughtered in an upright position was longer than for calves slaughtered in an inverted position. It was therefore decided, in order to minimize distress, to reduce the number of calves subjected to slaughter in an upright position, explaining the difference in sample size. The difference in time to loss of consciousness between the two treatments may be explained by poorer bleeding out of calves slaughtered in an upright position. Blood flow was rated in each calf as poor, moderate or good. In some of the calves slaughtered in the upright position, blood flow was obstructed when the position of the head temporarily restricted the flow of blood from the vessels in the neck. However, too few observations were available per rating to statistically support the difference between these two groups of cattle. Inversion of cattle is widely debated and thought to cause distress because if a: longer time interval from entering the restrainer to full restraint, discomfort from the inverted position, hypoxemia and rumen pressure on the diaphragm Von Holleben et al., 2010; Petty et al., 1994; Tagawa et al., 1994). Another possible

welfare issue is the aspiration of blood and refluxing gut content after the neck cut in unstunned animals (EFSA, 2003). Though the latter problem is mainly associated with the inverted position, it was also observed in unstunned calves in the upright position (Grandin and Regenstein, 1994; Gregory et al., 2009). A report by the Farm Animal Welfare Council stated that there is little evidence of any welfare advantages of inversion in terms of the speed and efficiency of the cut (FAWC, 2012). Although others claim, that the angle of the head in inverted cattle permits for a more effective downward cut compared to the upward cut when cattle are in an upright position (Slaughter of Animals (prevention of cruelty) Regulations 1958 – quoted by Dunn 1990). This study is the only one to suggest that unstunned animals slaughtered in an inverted position may benefit from the effectiveness of such a cut in time to loss of consciousness.

In 15 of the 82 (18%) calves in our study a second intervention was required, and for one of those 15 calves a third intervention was needed to secure bleeding. The arteries of an animal that is bleeding out poorly, should be re-cut immediately after observation to stimulate blood flow. Poor bleeding out may be caused by occlusion, which is characterised by retraction and contraction of the elastic portion of the arterial wall and thrombus formation around the cut end of the vessel (Gregory et al., 2006). Cattle are especially at risk of prolonged consciousness when occlusion of the severed carotid arteries occurs, since the vertebral arteries can supply blood to the entire bovine brain through the occipito-vertebral anastomosis (Johnson et al., 2014). The prevalence of carotid arterial occlusion in 576 cattle slaughtered at abattoirs in the United Kingdom was found to be 16% and 25%, respectively, for adult cattle and bobby calves (Gregory et al., 2006). A study by Gregory et al., 2010 showed that seventy-one percent of the cattle which took longer than 75 s to collapse had a false aneurysm in the cardiac end that was at least 3 cm in diameter. There was no investigation into an explanation for obstructed blood flow in this study, but this may explain why there was so much variation in time to loss of consciousness between calves.

In some of the calves, reflexes were lost and regained during bleeding. Loss and return of the cornea reflex were observed by Hoffman (1900) in an unspecified number of cattle (Hoffman, 1900 cited by Gregory et al., 2010. Newhook and Blackmore, 1982 later suggested that resurgences of consciousness can occur following unstunned slaughter, based on the amplitude and rhythm of EEG activity. Gregory and Shaw, 2000 also describe examples of drifting in and out consciousness as humans lose or regain consciousness due to brain injury or wake up from sleep. An explanation of this drifting in and out of consciousness is that in cases of loss of consciousness during haemorrhaging, the escape of blood can occur in two phases (Gregory, 2005). Detailed study of the EEG activity did not show signs of resurgence of consciousness in any of the calves.

The threat reflex was lost in all unstunned calves prior to unconscious based on EEG activity.

Observation and interpretation of the righting reflex and rhythmic breathing in this study appeared impossible or unreliable. Observation of the righting reflex was made impossible for animals due the mandatory restraint of the animals. Movements of the head can’t be distinguished between conscious reactions or reflexes and involuntary muscle contractions. The full body restraint in a closed restraining box makes it impossible to observe breathing movements of the chest or abdomen. Furthermore, during unstunned slaughter the trachea is cut together with the large blood vessels and distinction between rhythmic or normal breathing and pathological air flow is unreliable. Therefore, the validity of the righting reflex and spontaneous rhythmic breathing as parameters to determine unconsciousness can be questioned when using a restraining box as in this present study
Absence of the threat reflex at 40 s did not indicate unconsciousness. The rapid loss of the threat reflex can possibly be explained by the sudden drop in blood flow following the cut causing hypoxia of the brain cells involved (Bourquet et al., 2011). A similar event was observed with regard to the withdrawal reflex that was lost in all, but one calf, prior to loss of consciousness based on EEG activity. Absence of the withdrawal reflex at 40 s did not indicate unconsciousness. This calf may have lost the reflex after loss of consciousness due to the testing frequency of 20 s. The threat reflex was not observed at the moment of testing in this calf. Besides the veins and arteries, the knife also transects other blood vessels, skin, muscle, trachea, oesophagus, sensory- and motor nerves and connective tissue. Transecting these soft tissues will cause a major amount of neural impulses to travel to the brain. The massive stimulation of all sensory nerves after the neck cut can lead to a state of shock and

distress, experienced as painful, for the duration of consciousness. Calves, therefore most likely did not respond to another and milder source of pain (Johnson et al., 2012). However, opinions on this subject are divided and claims have been made stating that with a clean incision made with an exquisitely sharp knife, significant pain and distress are minimized (Grandin, 1994; Rosen, 2004). Until now, neurophysiological methodology has not provided the absolute answer to this issue. The eyelid reflex was lost in 94% of unstunned calves after loss of consciousness was observed and in 72% of unstunned calves after observation of minimal brain activity. As during anaesthesia, the eyelid reflex is known to persist for a relatively long time and similar results were found in unstunned slaughtered sheep (Gerritzen et al., 2014). Despite the fact that the eyelid reflex has been critically appraised by experts, its absence is still uniformly considered a reliable indicator of unconsciousness, unless the optic nerve is impaired, thus prohibiting a response (Gregory and Shaw, 2000). The cornea reflex was lost in 94% of unstunned calves after observation of loss of consciousness and in 75% of unstunned calves after observation of minimal brain activity. Earlier work (Lambooij et al., 2012, Newhook and Blackmore, 1982) revealed that calves lost the cornea reflex up to one minute after they were considered unconscious based on EEG activity and persisted in some calves after a flat EEG trace had been observed. These results support the idea that a positive cornea- or eyelid reflex alone does not necessarily indicate consciousness, since positive brain stem reflexes might occur on the basis of residual brain stem activity and do not distinguish clearly between consciousness and unconsciousness (Anil, 1991).

5 Conclusion

Unconsciousness was induced in calves with or without pre-stunning and measurement of reflexes was linked to EEG activity. Observation of reflexes to assess unconsciousness can readily be applied in daily practice, but EEG recordings are required to validate such reflexes.

Based on EEG activity it was apparent that all calves lost consciousness immediately or very quickly after captive bolt stunning. Loss of consciousness was confirmed by the absence of threat-, withdrawal,- cornea,- and eyelid reflexes at 5 s post stunning.

Post-cut captive bolt stunning results in immediate loss of consciousness and is as effective as pre-cut captive bold stunning.

In this study, a neck cut in an upright position resulted in a delay to loss of consciousness compared to neck cutting in an inverted position.

Righting reflex is not a valid or applicable parameter for assessment of unconsciousness while animals are being fully restrained. Since restraining is mandatory until the animals are unconscious, the righting reflex should not be used to determine level of (un)consciousness at slaughter.

Rhythmic breathing in fully restrained animals with a cut trachea is very limited therefore, rhythmic breathing is considered an impractical parameter for assessment of unconsciousness at slaughter.

In general, but specifically at 40 s after neck cutting, absence of the threat- or withdrawal reflex did not indicate unconsciousness. Moreover, the majority of the animals did not respond to pain stimuli following the neck cut when, based on EEG activity, they were considered to be conscious. The response to a threat is often difficult to assess due to the amount of blood flowing over the eyes of inverted animals and was observed to be absent in conscious animals based on EEG activity.

On the other hand, presence of the eyelid- or cornea reflex at 40 s did not confirm consciousness. Absence of the eyelid-, or cornea reflex reflected unconsciousness, but both appeared distinctly conservative parameters for confirmation of unconsciousness since they only disappeared after animals had been unconscious for a considerable period.

Considering the parameters chosen to assess unconsciousness at slaughter it can be concluded that;
the righting reflex and rhythmic breathing are difficult to determine under practical conditions,
the threat-, and withdrawal reflex (reaction to pain stimuli) are invalid parameters,

the eyelid-, and cornea reflex provide a very conservative confirmation, but when these are absent they are a true indicator of unconsciousness.

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Wageningen UR Livestock Research
P.O. Box 338
6700 AH Wageningen
The Netherlands
T +31 (0)317 48 39 53
info.livestockresearch@wur.nl
www.wageningenUR.nl/en/livestockresearch

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